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Detection of PCR Inhibitors in Cervical Specimens by Using the AMPLICOR *Chlamydia trachomatis* Assay

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To determine the susceptibility of AMPLICOR *Chlamydia trachomatis* PCR to inhibitory factors possibly present in cervical specimens, we obtained cervical specimens from 200 gynecology patients attending our outpatient clinic. The prevalence of *C. trachomatis* infection was 4.1%, as determined by cell culture. All AMPLICOR specimens were tested in one procedure as described by the manufacturer, and after the specimen was spiked with *C. trachomatis*, several other pretreatment protocols were used. Complete inhibition of the PCR was observed in 38 (19%) cervical specimens. Heat treatment at 95°C, freeze-thawing, or 10-fold dilution of the samples reduced the initial inhibition to 9, 16, or 9%, respectively. A combination of heat treatment and 10-fold dilution reduced the inhibition to 4% of the samples. A second specimen type (swabs inoculated in 0.2 M sucrose phosphate buffer [2SP]) was also evaluated. A 10-fold dilution of the spiked 2SP specimens resulted in an inhibition rate of 6%, which was comparable to that obtained by centrifugation of the 2SP specimen prior to processing. Furthermore, it was shown that the inhibition was not correlated with blood contamination. Processing the specimens on the day of collection or the day after resulted in a higher inhibition rate than did delayed processing (27.6 versus 15.5%, respectively). An inverse correlation was found between the concentration of *C. trachomatis* added to the sample and the rate of inhibition observed. The inhibition was partly correlated with the pH of the cervical mucosa. Decreased inhibition was found at pH values of ≥7.5. The effects of blood, pH, and delay in processing were all evaluated by using the AMPLICOR specimen. We conclude that the susceptibility of AMPLICOR *C. trachomatis* PCR to inhibiting factors in cervical specimens can be significantly reduced if the pretreatment procedure includes heat treatment or the use of 2SP transport medium. Also, a 10-fold dilution of the clinical specimen followed by heat treatment will largely prevent the inhibition of this PCR.

In most studies, the sensitivity of AMPLICOR PCR for the detection of *Chlamydia trachomatis* in cervical specimens has been at least comparable to that of cell culture (1, 3, 7, 9). Nevertheless, several investigators have detected false-negative PCR results and suggested that certain inhibiting factors present in the cervical specimen may compromise the sensitivity of the assay. These shortcomings of the assay should be overcome by optimization of the sample pretreatment procedure in order to neutralize inhibitors in the specimen (1, 2, 5, 7).

In the present study, we evaluated the susceptibility of the Roche AMPLICOR *C. trachomatis* PCR assay to inhibitory factors possibly present in cervical specimens by spiking samples with a known number of elementary bodies. Furthermore, to circumvent false-negative results due to inhibition, several sample pretreatment protocols were studied.

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MATERIALS AND METHODS

Patients and specimens. Cervical specimens were collected from 200 women visiting the outpatient clinic for gynecology at the University Hospital in Rotterdam, The Netherlands. Two cervical specimens per patient were obtained: one AMPLICOR specimen and one cell culture specimen. The AMPLICOR specimen was collected by using the STD swab specimen collection and transport kit (Roche Diagnostic Systems, Branchburg, N.J.). The specimen was taken according to the manufacturer's guidelines. The specimen used for cell culture was collected with a Dacron swab (Medical Wire and Equipment Co., Corsham, Wiltshire, United Kingdom) and placed into 0.2 M sucrose phosphate buffer (2SP). The specimens were stored at room temperature and sent to the laboratory within 4 h. The AMPLICOR sample was stored at 4°C prior to processing. The 2SP sample for cell culture was stored at 4°C, or at −70°C when not processed within 24 h after collection. Blood contamination of the AMPLICOR specimen was registered. The cervical pH was tested in situ with colorfast pH indicator sticks (Merck, Darmstadt, Germany).

AMPLICOR PCR. A protocol was developed to analyze PCR inhibition in cervical samples in a controlled manner. Cervical specimens were deliberately spiked with *C. trachomatis* serovar D prior to the PCR. The stock solution was stored at −80°C in small aliquots prior to use. The stock contained 1.5×10^4 inclusion-forming units (IFU) of *C. trachomatis* per ml. Aliquots of the AMPLICOR specimens were spiked with a concentration of 100 IFU per PCR and processed by one of the following procedures: (i) that prescribed by the manufacturer's guidelines, (ii) heat treatment at 95°C for 10 min prior to the PCR, (iii) freeze-thawing twice in liquid nitrogen prior to the PCR, (iv) pretreating the sample at 4°C, (v) a 10-fold dilution with AMPLICOR transport buffer prior to the PCR, or (vi) a 10-fold dilution in AMPLICOR transport buffer and subsequent heating of the sample at 95°C for 10 min prior to the PCR. Furthermore, the 2SP specimens were also spiked with a concentration of 100 IFU per reaction and processed by one of the following procedures: (i) specimens were diluted 10-fold with AMPLICOR transport buffer and processed as prescribed by the manufacturer, or (ii) 100 μl of 2SP specimen was centrifuged at 10,000 × *g* for 5 min at room temperature, resuspended in 100 μl of AMPLICOR transport buffer, and processed by a scaled-down version of the manufacturer's method for processing AMPLICOR swab specimens. Statistical evaluation of the collected data was performed by Fisher's exact test. Statistical significance was accepted at $P \leq 0.05$ (two-tailed).

TABLE 1. Effect of additional pretreatment procedure and transport medium on the inhibition of AMPLICOR *C. trachomatis* PCR assay

Specimen and additional pretreatment method	% of samples inhibited	P^a
AMPLICOR		
None	19.0	
Heating 10 min at 95°C	9.3	0.032
Freeze-thawing twice	15.7	0.535
10-fold dilution	9.3	0.041
10-fold dilution followed by heating 10 min at 95°C	4.1	0.0003
Pretreatment at 4°C	21.2	0.655
2SP		
10-fold dilution	5.8	0.0002
Centrifugation	5.8	0.0002

[a] Compared with AMPLICOR pretreatment protocol prescribed by the manufacturer's guidelines; measured by Fisher's exact test.

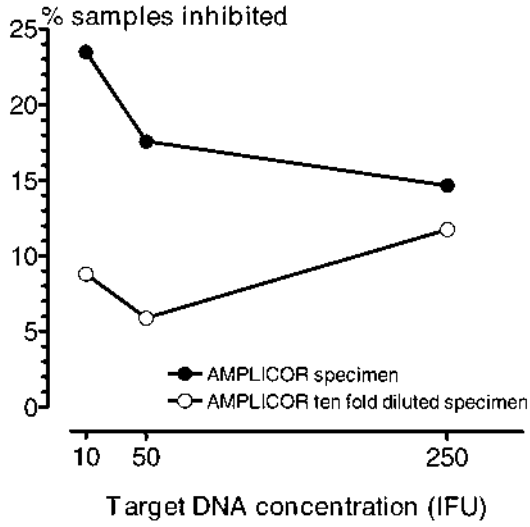

FIG. 1. Effect of target DNA concentration on the inhibition of AMPLICOR *C. trachomatis* PCR assay ($n = 34$).

RESULTS

Inhibition was observed in 38 (19.0%) of the cervical specimens when the AMPLICOR protocol was used as prescribed by Roche (protocol i). Heat treatment to 95°C for 10 min prior to processing (protocol ii) reduced the initial inhibition rate significantly to 9.3% ($P = 0.032$). Only one sample initially positive by PCR became negative with this pretreatment procedure. Freeze-thawing (protocol iii) resulted in no significant decrease in the initial rate of inhibition. (The inhibition rate was 15.7% [$P = 0.535$].) Three initially positive samples became negative with this particular treatment procedure. Also, adding cold specimen diluent to specimens and incubating them for 10 min at 4°C, instead of room temperature, did not change the inhibition rate significantly. (The inhibition rate was 21.2% [$P = 0.655$].) In contrast, a 10-fold dilution of the sample resulted in an inhibition rate of 9.3%, while a combination of heat treatment with a 10-fold dilution resulted in an inhibition rate of only 4.1% ($P = 0.041$ and 0.0003, respectively, compared with the initial inhibition rate found with the procedure prescribed by Roche) (Table 1). Eight initially inhibited samples became positive after this combined procedure, while none of the initially positive samples became negative.

The patient samples transported in 2SP were also spiked with *C. trachomatis* elementary bodies at a concentration of 100 IFU per PCR and tested by two different pretreatment methods. A 10-fold dilution of the 2SP specimen resulted in a low inhibition rate of 5.8%, which was comparable to the rate obtained by centrifugation of the 2SP specimen prior to processing of the sample ($P = 0.0002$, compared with the initial inhibition rate) (Table 1).

To gain insight into the nature of the inhibitory factor(s), 34 AMPLICOR specimens were spiked with either 10, 50, or 250 IFU of *C. trachomatis* per PCR before the PCR was carried out as prescribed by the manufacturer. It can be observed that the number of negative samples decreased as the number of target DNA molecules increased (Fig. 1). The inhibition rates were 23.5, 17.6, and 14.7%, respectively, with the procedure prescribed by Roche Molecular Systems. This dose-dependent effect was circumvented by 10-fold dilution of the specimens, which resulted in inhibition rates of 8.8, 5.9, and 11.8%, respectively.

Another factor which was taken into account was the stability of the inhibitory factor(s). One hundred sixty-eight specimens (84%) were processed within 7 days. Processing the specimens on the day of collection or the day after resulted in a higher inhibition rate than was obtained by delayed processing. The inhibition rates found were 27.6 and 15.5%, respectively ($P = 0.072$).

Since it is known that blood can be inhibitory to PCR, the blood-containing specimens were evaluated vis-à-vis the specimens without visible blood contamination. Thirty-two of 163 specimens (19.6%) without visible blood contamination were inhibited by PCR. Thirty-seven specimens were contaminated with blood; of these, 6 (16.2%) were inhibited, a rate not significantly different from that found in specimens without blood contamination.

Furthermore, the pHs of the cervical mucosae were registered for 126 patients to study if there is a correlation between the pH and the rate of inhibition. No significant correlation was found between the pHs of the cervical mucosae and the inhibition rate. Of 42 specimens taken from women with cervical pHs between 3.5 and 5.5, 9 (21.4%) were inhibitory for PCR. Of 69 specimens from women with cervical pHs between 5.5 and 7.5, 14 (20.3%) showed inhibition. Fifteen patients had cervical pHs of ≥ 7.5; for this group, the inhibition rate observed was reduced, but not significantly different, from that for the patient group with cervical pHs of <7.5 (6.7 and 20.7%, respectively; $P = 0.30$).

DISCUSSION

Failure to detect *C. trachomatis*-specific DNA by PCR in cervical specimens has been reported by others (1, 2, 4–8, 10–12). Some inhibitors could be removed by dilution or heat treatment of the sample prior to processing for PCR (4, 6, 8, 12). Also, prolonged storage of the AMPLICOR specimen reduced the inhibitory activity in several studies (2, 7, 10). The use of alternative transport procedures can increase the sensitivity of the PCR. 2SP has been reported to be an improved transport medium for AMPLICOR PCR (11). Also, freezing the sample in saline or resuspending a dried endocervical swab in saline has been reported to increase the sensitivity of AMPLICOR PCR (1, 5).

The aim of the present study was to determine the impact of possible PCR inhibitors in cervical specimens with the AMPLICOR *C. trachomatis* assay, with a view to further improve-

ment of the diagnosis of *C. trachomatis* infections by PCR. Significant inhibition was observed frequently in cervical specimens when the AMPLICOR protocol prescribed by Roche Diagnostic Systems was used. The inhibition was clearly inversely correlated with the target DNA concentration. This phenomenon was not observed if a 10-fold dilution of the sample was used, indicating a baseline inhibition which will remain present despite an increase in target DNA load.

The inhibitory activity found in our study can be reduced by delayed testing, which would indicate the presence of instable compounds, such as hormones and/or enzymes, that become inactive over time. This phenomenon has been confirmed by several other investigators (2, 7, 10). Also, the pH of the cervical mucosa may be partially responsible for the inhibition. Decreased inhibition was found at pH values of $\geq$7.5. A 10-fold dilution of the AMPLICOR specimen will increase the pH and therefore reduce the effect of pH. It can be concluded that PCR inhibitory activity in cervical specimens can be largely removed by heat treatment, a 10-fold dilution, or a combination of the two. Also, the use of 2SP as an alternative transport medium for the AMPLICOR *C. trachomatis* PCR assay has a similar effect. This can be explained by the pretreatment procedure. Using only the sediment after centrifugation or a 10-fold diluted sample will diminish the effect of all soluble inhibitory compounds.

Freeze-thawing twice prior to processing had only a minor effect on the outcome of the PCR. However, three initially positive specimens became negative. This could indicate that freeze-thawing destroys target DNA, resulting in decreased sensitivity of the PCR assay. DNase may also be responsible for this phenomenon.

In response to the results found in this study, it would be preferable to routinely include an internal control in each amplification mixture, which would greatly improve the quality of diagnosis of *C. trachomatis* infections.

The impact of these newly defined pretreatment methods can be observed only in a clinical study to determine the prevalence of inhibitory factors in *C. trachomatis*-positive patients. The specimens would have to be tested in a prospective manner to overcome major differences between the study and routine clinical practice. Batch processing of the specimens after storage can reduce the inhibitory effect, as was demonstrated in the present study. Many patients would have to be included to accomplish such a study. Since this is very labor-intensive, we decided to perform the present prospective controlled study.

We have studied the prevalence of *C. trachomatis* in patients attending the outpatient clinic for gynecology in a general hospital, Diakonessen, Utrecht, The Netherlands by using 2SP as an alternative transport medium for AMPLICOR PCR, and we have compared these results with those of cell culture. In this study, the sensitivities of AMPLICOR PCR and cell culture were 97 and 69%, respectively (data not published). In another ongoing study, we evaluated the COBAS AMPLICOR PCR for the detection of *C. trachomatis* and *Neisseria gonorrhoeae* in urine and compared the results with those of cell culture. An internal control was incorporated in this evaluation. At the time the data reported in this article were tabulated, 1,000 patients were enrolled in this study. Twenty-six patients had results indicating inhibition of the PCR. Only one specimen remained inhibited after being heated at 95°C for 10 min. Two patients became positive for *N. gonorrhoeae* after successful PCR processing. No inhibition was found when a 10-fold dilution was used, although the two positive *N. gonorrhoeae* specimens became negative after dilution, indicating a reduction in sensitivity.

We conclude that AMPLICOR *C. trachomatis* PCR for cervical specimens can be significantly improved if the pretreatment procedure includes heat treatment or the use of 2SP transport medium. A 10-fold dilution of the clinical specimen followed by heat treatment will also decrease PCR inhibition significantly.

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